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Full Length Research Paper

Characterization of a cinnamoyl-CoA reductase gene in *Ginkgo biloba*: Effects on lignification and environmental stresses

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Cinnamoyl-CoA reductase (CCR, EC 1.2.1.44) catalyzes key steps in the biosynthesis of monolignols, which serve as building blocks in the formation of plant lignin. The full-length cDNA of *GbCCR* is 1178 bp and contains a 972 bp open reading frame (ORF) encoding a 323 amino acid protein. The deduced GbCCR protein showed high identities with other plant CCRs, and had closer relationship with *Picea abies*, sharing 56.3% homology. They both contain a common signature which is thought to be involved in the catalytic site of CCR. Phylogenetic tree analysis revealed that *GbCCR* shared the same ancestor with other CCRs, but the divergence time is early. Southern blot analysis indicated that *GbCCR* belonged to a multi-gene family. The expression analysis by quantitative real-time polymerase chain reaction (QRT-PCR showed that *GbCCR* was seen in a tissue specific manner in *Ginkgo biloba*; it had the highest expression in injured stems, and a high expression in four years old stems, while it had the lowest in endosperm. *GbCCR* was also found to be significantly up-regulated by gibberellin (GA), but the expression was weakly induced by *Agrobacterium* treatment. QRT-PCR analysis showed that *GbCCR* activity correlated with changes in transcription level of the *GbCCR* gene, and *GbCCR* activity was also positively correlated with total lignin accumulation in developments of *Ginkgo* stem. In light of these properties and expression pattern, we suggested that the corresponding enzyme is probably involved in constitutive lignification and defense.

Key words: *Ginkgo biloba* L., *GbCCR*, gene expression, lignification, defense.

INTRODUCTION

The phenylpropanoid pathway produces a wide range of secondary metabolites, flavonoids, anthocyanins, isoflavonoids, silbenes and lignins (Bayindir et al., 2008). Lignin is the second most abundant natural organic substance after cellulose (Whetten et al., 1998). It is normally located in the secondary cell walls or intertwined with cellulose to confer structural rigidity to plants. In addition, its hydrophobic properties make the cell wall water-impermeant, facilitating long-distance water and solute transport inside the plant. Finally, lignin acts as a physical barrier, preventing the invasion of pathogenic bacteria (Baucher et al., 1998).

Lignin is the product of the polymerization of three monomers: syringyl propane, guaiacyl propane and hydroxyphenyl propane, and can be classified into three types: syringyl lignin (S-lignin), guaiacyl lignin (G-lignin) and hydroxyphenyl lignin (H-lignin) (Freudenberg, 1959). The three lignin monomers are synthesized through distinct pathways, but the reduction of precursors by cinnamoyl-CoA reductase (CCR; EC 1.2.1.44) and dehydrogenation by cinnamyl alcohol dehydrogenase (CAD) are key steps in the synthesis of all three.

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Cinnamoyl-CoA reductase (CCR, also called feruloyl-CoA reductase, p-hydroxycinnamoyl coenzyme A reductase or cinnamoyl-CoA: NADPH reductase) is the rate limiting enzyme in the biosynthesis of lignin monomers (Lacombe et al., 1997) and is thus a potential control point in the regulation of carbon flow towards lignin synthesis (Piquemal et al., 1998; Jones et al., 2001). Electro- phoresis of the purified enzyme and informatics analysis of cloned genes indicates that the molecular weight of CCR is generally above 30 kDa, but varies slightly between plant species (Lüderitz and Grisebach, 1981). It is not yet known if any CCR protein have a signal peptide, and the intracellular distribution has not been determined. The basic consistency between the theoretical molecular weight of the *CCRs* gene encoded protein and the molecular weight of purified CCRs protein, however, indicates that CCR is a single subunit enzyme. After translation, there are no apparent post-translational modifications that alter the peptide chain or the molecular weight (Sarni et al., 1984).

At present, *CCR* genes have been cloned from *Pyrus pyrifolia*, *Zea mays*, *Arabidopsis thaliana*, *Lolium perenne*, *Oryza sativa*, *Populus tomentosa*, *Isatis indigotica*, *Eucalyptus globulus* and *Pinus massoniana* (Pichon et al., 1998; Lauvergeat et al., 2001; McInnes et al., 2002; Bai et al., 2003; McKinnon et al., 2005; Bihua, 2009; Wang et al., 2009; Lu et al., 2010; Hu et al., 2011). The *CCR* gene was firstly isolated from *Eucalyptus gunnii* and had a correlation with tissue lignification. Recent studies indicate that CCR is a key enzyme for the control of lignin monomer synthesis, so changes in the expression of lignin synthesis genes will determine the accumulation of lignin in different plant tissues. Their functions and features have been studied in detail. In *Eucalyptus*, *CCR* is mainly distributed in xylogen and thick cell walls (Goffner et al., 1994). In *Pyrus pyrifolia*, *CCR* gene is involved in the regulation of lignin biosynthesis and plays an important role in stone cell formation (Lu et al., 2010). During the growth of plant stems, *CCR* participates in the biosynthesis of lignin that is critical for enhancing stem strength during elongation (Ma, 2007; Tu et al., 2010). Other researchers noted that CCRs have a different expression pattern; in *Panicum virgatum,* PvCCR1 is probably associated with lignin biosynthesis during plant development and PvCCR2 may function in defense (Escamilla et al., 2010). Lauvergeat (2001) suggested that AtCCR2 responsive to pathogen infection of *Xanthomonas campestris* may play a role in the formation of phenolic compounds associated with hypersensitive response in *A. thaliana*. It has been suggested that the CCR mediated reaction products of monolignols are not only used for polymerization into lignins, but into others such as antioxidants or biocides (Lacombe et al., 1997).

Here, we identify a cDNA similar to *CCRs* in *Ginkgo biloba*. A combination of biochemical and gene expression analysis indicated that GbCCR is the enzyme involved in lignification and defense. Hence, the cloning of *GbCCR* genes will facilitate the study of *CCR* gene regulation during development and under different environmental conditions. In turn, manipulation of *CCR* and other genes in the phenylpropane pathway could facilitate the development of *Ginkgo* plants enriched in specific flavonoid compounds or plants of greater size and *Ginkgo* timber quality.

MATERIALS AND METHODS

12-years old grafted *G. biloba* seedlings grown in a greenhouse in Huanggang (E, 114°54'-116°8', N, 29°45'-31°35', Hubei province, central China) were sampled as plant gene isolation materials. For gene cloning and tissue expression, diverse tissues including young leaves, mature leaves, ovules, stamens, albumen, gynoecia, stems and roots were collected for DNA and RNA extraction as described by Xu (2008). Tissues were immediately frozen in liquid nitrogen and kept at -80°C prior to total RNA extraction.

Total RNA was isolated from injured stem tissues of 12-years old grafted *G. biloba* seedlings after they were attacked by *Rhizoctonia solani*. For fungal infections blade, the total RNA was isolated from *Ginkgo* leaves cells punctured with an infected agent isolate of *Phyllosticta mortoni Fairman*.

A suspension culture of *Ginkgo* cell line was used for *Agrobacterium tumefaciens* infection stress treatments. Picked saved *Agrobacterium* LBA4404 containing a single colony was inoculated into the rifampicin (Rif, 40 mg/L) activation of the yeast extract peptone (YEP) culture medium to grow to logarithmic phase (OD600 = 0.5), centrifuged to collect the right amount of *Agrobacterium* with 50 ml MS liquid medium (MS + 1 mg/L NAA + 0.05 mg/L KT; pH adjusted to 7.0), re-suspension of bacteria was adjusted to OD600 = 0.5. The suspension cell of *Ginkgo* was collected by centrifugation from a standing liquid medium and inoculated into a fresh liquid medium in LBA4404; the inoculated suspension cells (4 g) were then put in 50 mL bottle as control. This sample was co-cultured for 2, 4, 6 and 8 h and the suspension cells were collected after extraction of RNA, through quantitative real-time polymerase chain reaction (QRT-PCR) identification of *GbCCR* gene expression.

Ginkgo suspension cells were collected in the same bottle of 50 ml of fresh liquid medium inoculated with cells in the control of 4 g, adding sterile gibberellic acid (GA3) to a final concentration of 0.1 mmol/L. When coculture was done for 4, 8, 12, 48 and 96 h, suspension cells were collected after extraction of RNA, by QRT-PCR identification of *CCR* gene expression.

Molecular cloning of the *GbCCR* cDNA

Given species conservation of the functional domain of the CCR protein, a pair of degenerate primers, CCRF and CCRR, was designed according to the conserved peptide sequences, RVVFTSSI and NWYCYAK, to amplify a conservative fragment of the *GbCCR* gene. The purified PCR product was cloned into the pMD18-T vector (TaKaRa, Dalian China), and the positive clone was confirmed by a BLASTN query of the NCBI Database to verify the homology of this cloned fragment with *CCR* sequences from other species.

Based on the sequence of cloned *GbCCR* fragments, the specific primer pairs (CCR5R and CCR3R) and the nested primer pairs (CCR5N and CCR3N) were designed to amplify the 5' and 3' end of *GbCCR* using the SMART™ RACE cDNA Amplification Kit (Clontech, USA). The PCR products were purified and cloned into the pMD18-T vector for sequencing. After comparing and aligning

Table 1. Primers for *GbCCR*.

Primer	Sequence (5'-3')	Description
CCRU	CGYGTGRTGTTAACWTCTNCADTTG	Degenerate primer, forward
CCRD	CTCTSCCACTGKCTTTGCHTAGCNATAC	Degenerate primer, reverse
CC5R1	AACGAGAGCATCAGGATTTCTGTTGGG	Reverse primer for 5'RACE, outer
CC5R2	ACAGCCCCAATTGAAGATGTTA	Reverse primer for 5'RACE, nested
CC3R1	ATGAACCCCAACAGAAATCCTGATGCT	Forward primer for 3'RACE, outer
CC3R2	CTGGAGCGATCTGGATTACTGC	Forward primer for 3'RACE, nested
CCRZ1	*ACAGGATCC* ATGGATTGCAATGGAGTAGAAGA	Gene-specific primer, forward
CCRZ2	*CAACTCGAG* CTAGTGAAGAAAACCCTTTTCCT	Gene-specific primer, reverse
CCT1	GCTCTCTGCTATTTATGGCTGC	Gene-specific primer, forward
CCT2	ACTGCTCTTGATTGGCGTGAAC	Gene-specific primer, reverse
CCT3	CCTTGCCCATTTGTTTGT	Gene-specific primer for QRT-PCR
CCT4	CACCCTTTCCTTCGTATCTT	Gene-specific primer for QRT-PCR
GAPU	TAGGAATCCCGAGGAAATACC	Primer for QRT-PCR, forward
GAPD	TTCACGCCAACAACGAACATG	Primer for QRT-PCR, reverse

Restriction site sequences are indicated in italic letter and underlined.

the sequence of 5'RACE, 3'RACE and the internal fragment, the full-length cDNA sequence of *GbCCR* was obtained.

Southern blot analysis

Genomic DNA (20 µg/sample) was digested overnight at 37°C with *Eco* R I, *Bam* H I and *Hin*d III. The digested DNA was fractionated by 0.9% agarose gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane (Roche Applied Science, Germany). Specific probes were generated by PCR: GbCCR specific probe was obtained using sense CCT1 and antisense CCT2 primers located respectively at positions 280 and 958 on the *GbCCR* cDNA. A total of 50 ng purified amplified fragments were used as a template in a total volume of 20 µl for probe labeling. Probe labeling (DIG), hybridization and signal detection were performed following the manufacturer's instruction for the DIG High Primer DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany) (Cheng et al., 2011).

Expression of GbCCR recombinant proteins in *Escherichia coli*

To clone *GbCCR* into an expression vector, a pair of primers and were designed and synthesized to amplify the coding region by QRT-PCR with the incorporation of a restriction enzyme site and a protective base to simplify later vector construction. After confirmation by sequencing, the resulting recombinant plasmid was introduced into BL21 (DE3) by the heat shock method. A single colony of *E. coli* BL21 cells harboring the expression plasmid pET28a-GbCCR was inoculated at 37°C in Luria-Bertani medium containing kanamycin (50 mgL^{-1}) and was grown with shaking (160 rpm) at 37°C until the optical density (OD600) reached about 0.55. For induction, isopropyl β-D-thiogalactoside (IPTG) was added at a final concentration of 1 mM and the cells were further cultured at 30°C for 2 h. The cells were lysed by sonication for 10 s at 4°C and centrifuged at 7000 g for 15 min. Supernatants and pellets were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue R250 staining. The recombinant GbCCR protein from induced cells was purified using Nickel-CL agarose affinity chromatography (Bangalore Genei). Protein concentrations were determined by the Bradford (1976) assay.

Western blotting was carried out to verify expression of a GbCCR protein having a His-tag in the N-terminus. After electrophoresis, the proteins were electrotransferred onto a PVDF membrane and detected with antimouse RGS-His antibody (Santa Cruz, American), and a secondary antibody (goat anti-mouse IgG), conjugated to alkaline phosphatase (AP). Western Blue Stabilized Color Substrate for AP (Promega, USA) was used for the color reaction.

Relative quantification by QRT-PCR

The transcription levels of *GbCCR* were determined in different *G. biloba* tissues, as well as in young seedling leaf samples collected at different time points after stress and hormone treatments. QRT-PCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, American) with SYBR Green PCR Master Mix (Applied Biosystems, American) according to the manufacturer's protocol. The *G. biloba* glyceraldehydes-3-phosphate dehydrogenase gene (*GbGAPDH*, L26924) (Jansson et al., 1994), was used as the reference gene as described by Xu (2008).

The gene-specific primers (CCT3, CCT4) and reference primers (GAPU and GAPD) for QRT-PCR are listed in Table 1. The QRT-PCR conditions were: 10 min at 95°C, and 40 cycles (95°C for 15 s, 60°C for 1 min). Before performing QRT-PCR, primer efficiency was evaluated using both *GbCCR* and *GbGAPDH* at 100, 150, 200, 250 and 300 nM combinations. A 150 nM concentration was chosen as most suitable combination for both genes. For each plant sample, aliquots of 150 ng total RNA was analyzed for each gene and the two genes (*GbCCR* and *GbGAPDH*) were also analyzed simultaneously. Each sample was amplified three times and all reactions were performed on ABI PRISM 7500 Sequence Detection System. With a housekeeping gene *GbGAPDH*, the relative amount of the *GbCCR* transcript is presented as $2^{(-ddCt)}$ according to the C_T method ($dCt = Ct_{sample} - Ct_{control}$) described in the QRT-PCR application guide (Applied Biosystems). When comparing the expression of *GbCCR* in different tissues, the relative expression of *GbCCR* was achieved by calibrating its transcription level to that of the reference gene, *GbGAPDH*.

Figure 1. The full-length cDNA sequence and deduced amino acid sequence of *GbCCR* gene. Start codon, ATG; stop codon, TAG.

Enzyme extraction and assay

The recombinant *GbCCR* activity was measured spectrophotometrically and determination of kinetic parameters was performed as previously described in Goffner et al. (1994). The three substrates: feruloyl-CoA, sinapoyl-CoA and caffeoyl-CoA, were synthesized according to the method of Stöckigt et al. (1975). For each reaction, 12 min of declination at OD_{366} was monitored automatically with 1 min intervals. A non-induced *E. coli* extract was used as a control. Km and Vmax values were determined by extrapolation from Lineweaver-Burke plots.

Ginkgo different tissues and treatment sample were collected and ground under liquid nitrogen. The powdered tissues were extracted for 1 h at 4°C in extraction buffer (100 mM Tris-HCl pH 7.5, 0.2 mM $MgCl_2$, 2 mM DTT, 10% glycerol). The samples were spun at 12 000 g for 10 min at 4°C and the resulting supernatant was desalted on PD-10 columns (Pharmacia, USA). The soluble protein fraction was used for determination of CCR enzyme activity. Total CCR activity was measured by using the same protocol as used in the experiments by using the recombinant proteins.

Lignin content analysis and histo-chemical lignin staining

Lignin content was quantitatively measured by using the Klason method (Kirk and Obst, 1988). Briefly, air-dried stem tissues were ground into powder and exhaustively extracted in a Soxhlet apparatus with toluene-ethanol (2:1, v/v), followed by 95% ethanol and water. Samples were then vacuum dried and 200 mg was hydrolysed in 3 ml of 72% H_2SO_4 at 25°C for 3 h with occasional stirring. The hydrolysate was diluted with the addition of 190 ml H_2O and then autoclaved for 1 h. The sample was filtered through a fritted glass crucible, and then washed with hot water. The crucible was dried at 105°C and weighed. The filtered solution was diluted to 500 ml and A_{205} was determined spectrophotometrically using a 1 cm long cuvette. The Klason lignin was expressed as percentage of the cell wall residue (CWR). The acid-soluble lignin was calculated with the following formula:

Acid lignin ($g \cdot L^{-1}$) = A_{205}/110

For lignin staining observation, hand-cut sections of different sample were treated by 1% phloroglucinol ethanol and 35% HCl, and images were captured by Cannon camera (Lu et al., 2010).

Bioinformatics analysis and molecular evolution analyses

Similarity search of GbCCR proteins were performed with the Blastx or Blastp program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Multiple sequence alignment of deduced GbCCR with other CCR proteins was conducted using the Clustlx program. The phylogenetic tree was constructed by a neighbor-joining (NJ) method and measured by bootstrap analysis with 1000 replicates. Phylogenetic tree analysis of *GbCCR* and known CCRs from other plant species retrieved from GenBank were aligned with Mega 4.0 (Tamura et al., 2007). Vector NTI Suite 10 was used for sequence alignment and analysis. SPSS 17 was used for statistical analysis and graphing.

RESULTS

Cloning and sequence analyses of a gene encoding cinnamoyl-CoA reductase from *Ginkgo biloba*

Using the rapid amplification of cDNA ends (RACE) method and primers mentioned above, cDNA ends of 457 and 839 bp were amplified by 3' and 5'-RACE, respectively, and core sequence of 780 bp was amplified as well (Figure 1). These sequences above were assembled with Vector NTI Suite 10.0 and the full-length *GbCCR* cDNA was subsequently amplified by proof-reading PCR amplification with primers mentioned above.

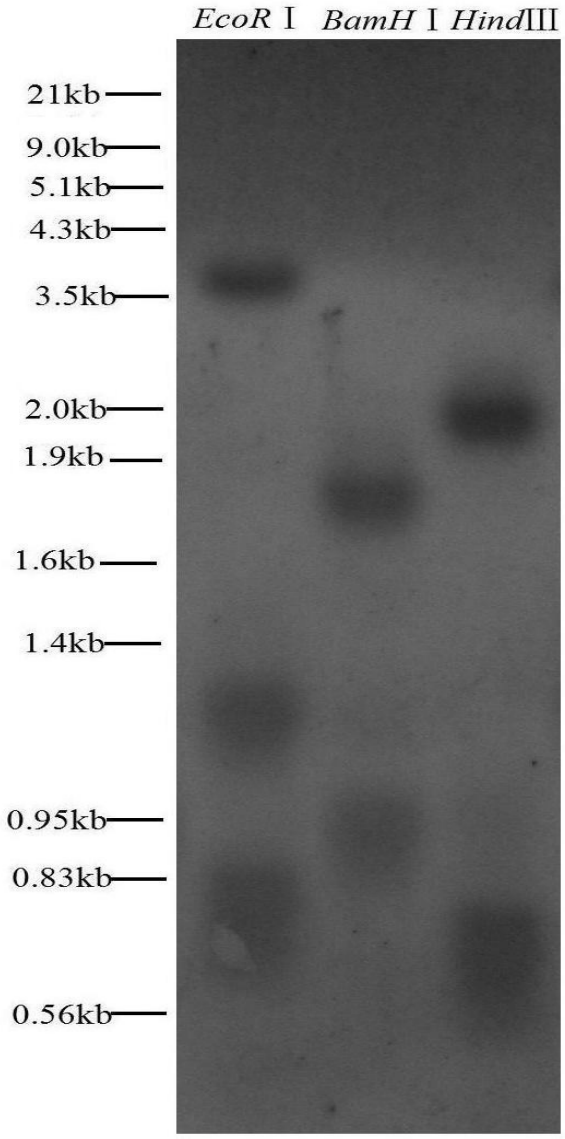

Figure 2. Genomic blot analysis of GbCCR. Genomic DNA was digested with *Eco*RI, *Bam*HI and *Hind*III. The DNA blot was hybridized with the insert in the *GbCCR* cDNA clone. Positions of molecular weight markers are shown on the left.

The full-length cDNA of CCR was 1178 bp with 55 bp 5' and 154 bp 3' untranslated regions, 29 bp poly A tail and contained a 972 bp ORF encoding a 323 amino acid protein. Analysis shows that the start codons of *GbCCR* sequences is TACAATGG, which is similar to the consensus sequences around the translation start codons (AACAATGG) in plants respectively (Lütcke et al., 1987). Southern blot analysis was carried out to investigate the genomic organization of *GbCCR* gene. Aliquots of 20 µg genomic DNA were digested with *Eco*R I, *Hin*d III and *Bam*H I, which did not cut within the coding region. These fragments were then hybridized with the coding sequence of GbCCR under high stringency conditions. As shown in Figures 2 and 3, specific hybridization bands ranging from 0.5 to 15 kb were recovered in each of the restriction enzyme-digested lanes; this indicated that *GbCCR* belonged to a multigene family (Figure 2).

Characterization of the deduced GbCCR protein and phylogenetic tree

By using the vector NTI 10 software and Swissmodel, the calculated isoelectric point and molecular weight of the deduced GbCCR were predicted to be 7.2 and 35.94 kDa, respectively. Multiple sequence alignments of amino acid showed that two highly conservative regions were identified, the common signatural CCR catalytic site NWYCYAK and the N-terminal portion that contains 21 amino acid residues sited at positions 15 to 35. This particular region was thought to form the β-α-β structure and was the putative NADP binding domain (Lacombe et al., 1997). The amino acid sequence of the GbCCR shared 68.6% identity with that of *Picea abies* (CAK18610), 68.0% with *Solanum tuberosum* (AAN71761), 67.0% with *Eucalyptus gunnii* (CAA56103), 68.3% with *Linum album* (CAD29427), 67.9% with *Betula luminifera* (ACJ38670), 68.0% with *Codonopsis lanceolata* (BAE48787), 66.7% with *Eucalyptus urophylla* (CBG37721), 66.1% with *Isatis tinctoria* (ADC40029), 67.1% with *Populus trichocarpa* (EEE84065), 63.2% with *Hordeum vulgare* (AAN71760), 62.5% with *Z. mays* (ABF24616) and 65.4% with *A. thaliana* (NP107754) (Figure 3A).

A phylogenetic tree generated by the neighbor-joining method based on the putative amino acid sequences of CCRs showed that all members of the CCR family could be sorted into three monophyletic group. The *G. biloba* CCR was included in a polyphyletic group with other CCRs from various groups (Figure 3B).

Expression of CCR in *E. coli*

In order to express GbCCR in *E. coli*, we cloned the coding sequence of *GbCCR* into pET-28a, an expression vector with the T7 promoter and a His-tag, yielding pET28a-*GbCCR*. The expression construct was checked for in-frame fusion by DNA sequencing (Sangon, China). Upon induction by IPTG, GbCCR was expressed as a major soluble protein product. The molecular weight of the expressed recombinant protein was estimated as a 36 kDa band with a His-tag: this size was in agreement with the one predicted by Vector NTI 10.0. The recombinant GbCCR protein was purified using Nickel-NTA agarose affinity chromatography and the purified protein showed the expected size (Figure 4). Western blotting of purified recombinant GbCCR protein confirmed its specific immune reactivity to anti-His antibodies.

GbCCR protein was purified to homogeneity from the soluble fraction of induced *E . coli* extracts and its kinetic

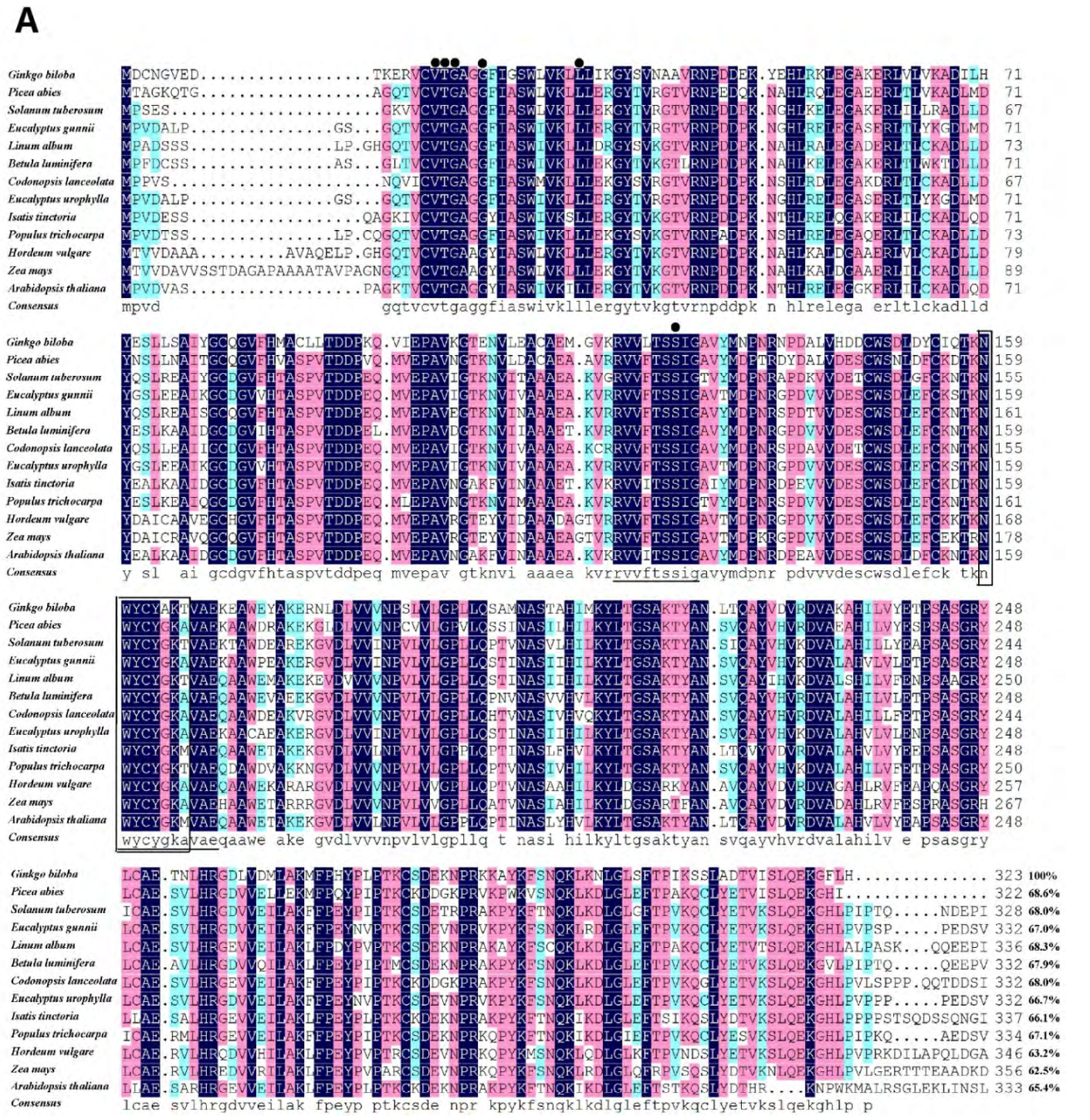

Figure 3. Multiple alignment and phylogenetic tree of the deduced amino acid sequences of GbCCR with CCRs from other plant species. (A) Alignments of sequences were performed using the Clustal W program on the EBI WWW molecular biology server. Sequences used for designing PCR primers are underlined; Residues of the NADPH binding marked with solid round;the substrate binding and the residues of active site indicated box. The accession numbers of the sequences used in the comparison are: *Arabidopsis thaliana,* NP107754; *Picea abies,* CAK18610; *Jatropha curcas,* ACS32301; *Pinus massoniana,* ACE76870; *Camellia oleifera,* ACQ41893; *Solanum tuberosum,* AAN71761; *Oryza sativa,* CAD21520; *Solanum lycopersicum,* AAY41880; *Eucalyptus gunnii,* CAA56103; *Linum album,* CAD29427; *Betula luminifera,* ACJ38670; *Eucalyptus globulus,* AAT74879; *Leucaena leucocephala,* ACB45309; *Lolium perenne,* AAG09817; *Codonopsis lanceolata,* BAE48787; *Eucalyptus urophylla,* CBG37721; *Isatis tinctoria,* ADC40029; *Leucaena leucocephala,* CAK22319; *Populus trichocarpa,* EEE84065; *Hordeum vulgare,* AAN71760; *Populus tremuloides,* AAF43141; *Vaccinium corymbosum,* ACI14382; *Triticum aestivum,* AAX08107; *Zea mays CCR,* ABF24616; *Zea mays DFR,* CAA48207. (B) The tree was constructed using the Neighbor-Joining method, MEGA4.1. The numbers at each node represent the bootstrap value, with 1000 replicates. The accession numbers of the sequences used in the comparison are: CAK18610 (*Picea abies*) and ACE76870 (*Pinus massoniana*).

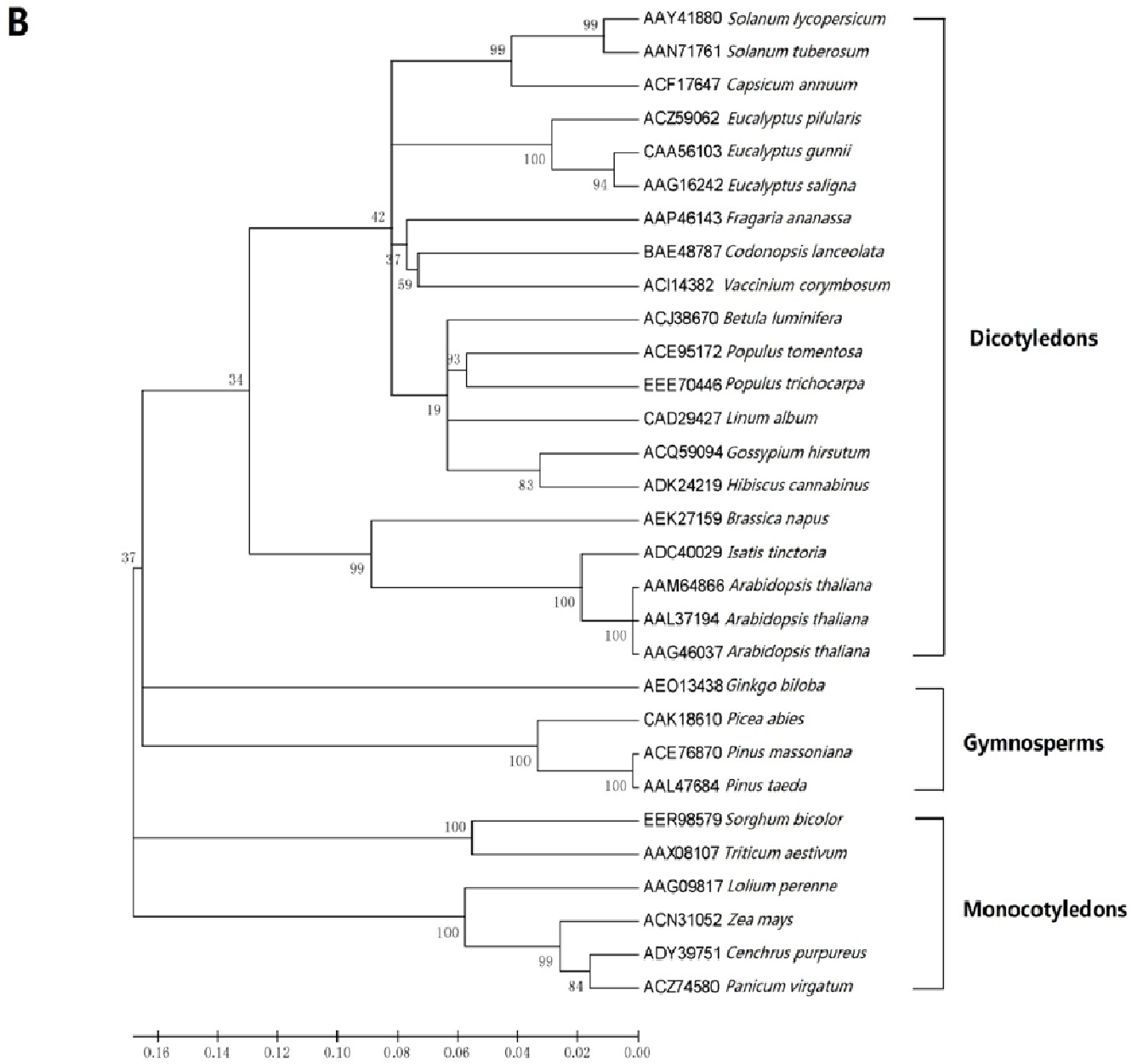

Figure 3. Contd.

parameters were determined (Table 2). The calculated catalytic efficiency (K_{cat}/K_m) of GbCCR indicated that feruloy-CoA was the best substrate, in consideration of its low K_m (37.26 ± 5.4 μM) and high V_{max} (198.51 ± 33.27 nkat·mg^{-1}protein) values relative to the other substrates tested. The catalytic efficiency of the other three substrates was lower than that of feruloyl-CoA. Compared with feruloyl-CoA, caffeoyl-CoA had a similar V_{max} value (156.48 ± 4.93 nkat·mg^{-1} protein), but higher K_m values (52.26 ± 3.6 μM), while sinapoyl-CoA had lower K_m values (14.73±8.2 μM) with correspondingly lower V_{max} values (18.21 ± 3.4 nkat·mg^{-1}protein).

Expression of *GbCCR* gene in relation to lignin synthesis in different tissues

In order to determine if *GbCCR* gene was related to lignin synthesis in *Ginkgo*, the expression pattern of *GbCCR* was analysed by QRT-PCR. Here, we found that *GbCCR* was expressed in the stems, leaves, roots, fruits, pistil and stamen of *Ginkgo*. CCR is generally constitutively expressed, but greatly varies in different tissues (Figure 5). *GbCCR* has the highest expression level in stems, but the expression levels also significantly vary in different development stages (Figure 6). The highest level of

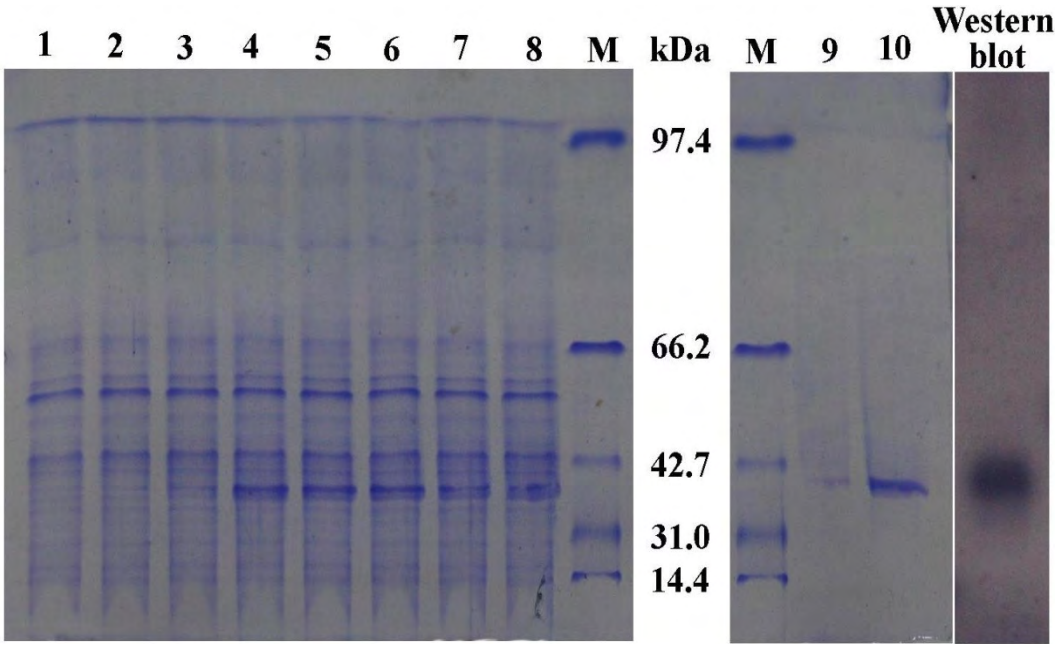

Figure 4. SDS-PAGE gel and Western blot analysis of GbCCR expressed in *E. coli* BL21 (DE3). After IPTG induction, *E. coli* BL21 cells containing pET28a-CCR were grown at 30°C for 2 h. M, molecular marker; lane 1, protein of total cells without IPTG induction; lane 2, protein of total cells with IPTG induction for 10 min; lane 3, protein of total cells with IPTG induction for 30 min; lane 4, induction for 1 h; lanes 5 and 6, induction for 1.5 h; lanes 7 and 8, insoluble proteins with IPTG induction for 2 h; lanes 9 and 10, purified recombinant GbCCR protein with nickel-CL agarose affinity chromatography used for enzyme activity assay. Lane western blot, western blotting of the purified recombinant GbCCR protein with an anti-His-tag primary antibody probe.

Table 2. Kinetic parameter of GbCCR protein.

Substrate	Km(μM)	Vmax(nkat·mg^{-1} protein)	Kcat/Km (min^{-1}μM^{-1})
Feruloyl CoA	37.26±5.4	198.51±33.27	10.7
Sinapoyl CoA	14.73±8.2	18.21±3.4	2.3
Caffeoyl CoA	52.26±3.6	156.48±4.93	7.4

Values represent the mean of three independent replicates ±SD.

transcription is found in the damaged part of the third-year stem. This was followed by the fourth-, third- and second-year stems having normal growth and infected by fungus. The lowest level of expression is found in first-year stems. The *CCR* gene expression level positively correlates with the accumulated level of lignin in corresponding parts. The damaged three-year stem has the most accumulated lignin, followed by undamaged fourth-, third-, second- and first-year stems. The fungus-infected parts of the second-year stem have more accumulated lignin in local parts, but the leaf stalk of *Ginkgo* has a low lignin accumulation level. Correspondingly, the CCR transcription level and enzymatic activity are quite low. Leaves have low lignin content, which is mainly concentrated in vein tissue. Infected leaves have apparently higher lignin content, consistent with the transcription and expression level of the *CCR* gene. In *Ginkgo* seeds, lignin is only synthesized in mesosperm, and there is almost no lignin in the endosperm. This finding is consistent with the transcription level of the *CCR* gene and lignin accumulation in endosperm. However, the expression level of the *GbCCR* gene and lignin accumulation in testa (mesosperm) has great differences (Figures 5 and 6).

Regulation of *GbCCR* gene transcription by *A. tumefaciens* (At) and GA

Meloidogynosis *A. tumefaciens* could infect the cells in the damaged parts of plants and cancerous cells. In the current experiment, suspended culture cells of *Ginkgo* embryo were inoculated by *A. tumefaciens* to induce *CCR* expression in *Ginkgo*. Two hours after inoculation, the expression level of *CCR* slightly dropped, then rose after

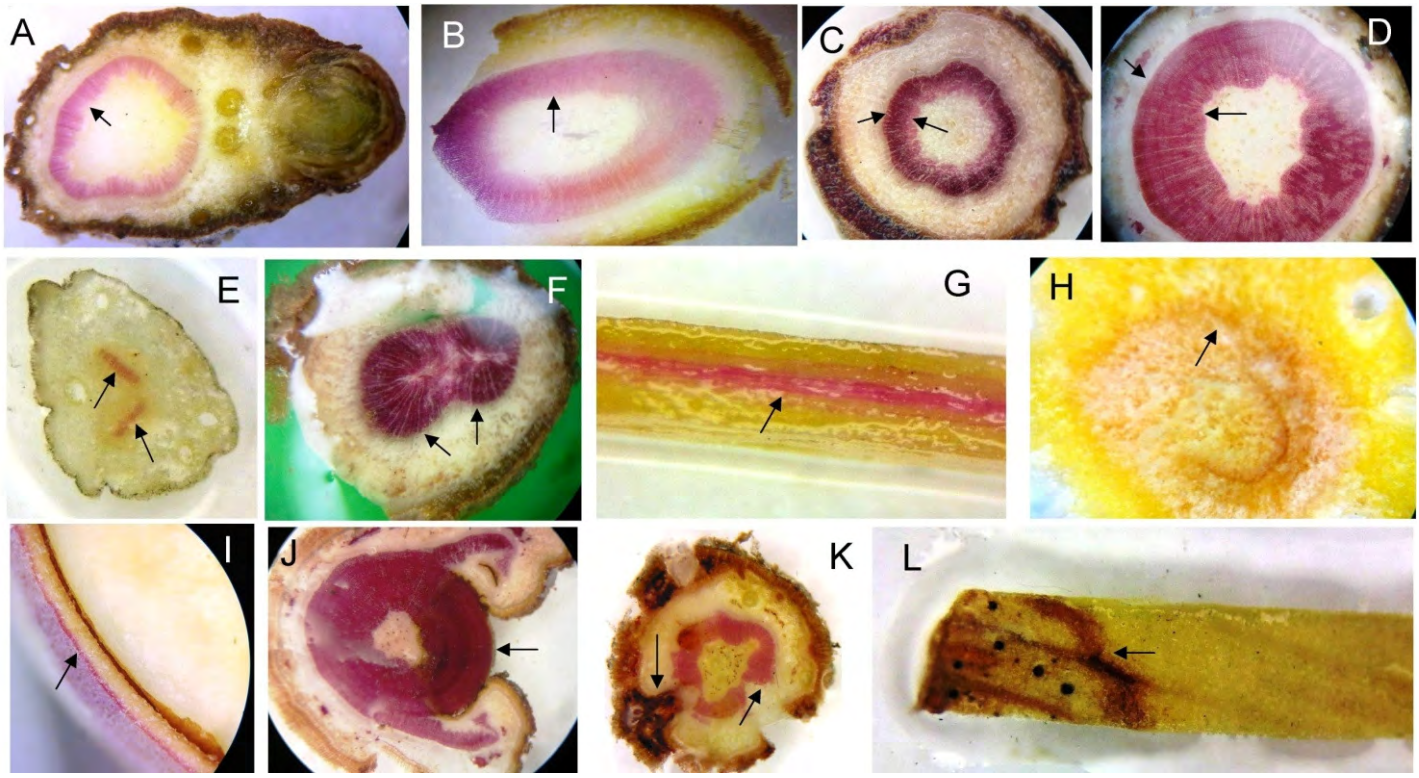

Figure 5. Change of lignin content in *Ginkgo* different tissues, development stage and environment. Sections staining with phloroglucinol-HCl. A, Annual stem; B, biennial stem; C, triennial stem; D, four years stem; E, petiole. F, root. G, leaf veins of the longitudinal cutting; H, endosperm; I, test; J, injured stems; K, diseases stems; L, fungal infections blade. The arrow indicates histo-chemical lignin staining.

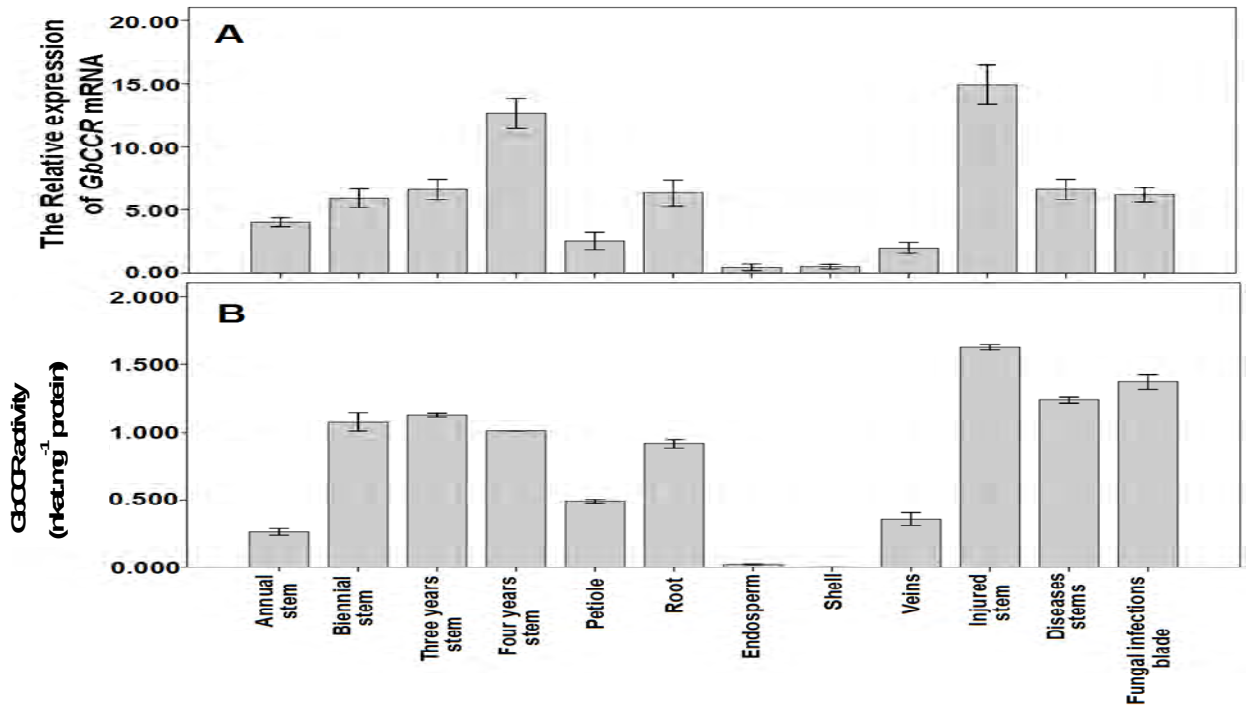

Figure 6. Comparison among different samples in *G. biloba*, regarding relative amount of *GbCCR* mRNA and enzyme activity of GbCCR. (A) Expression pattern of *GbCCR* gene in different tissues with GAPDH as control. (B) GbCCR activity in different sample. Data are mean values of triplicate tests ± SD.

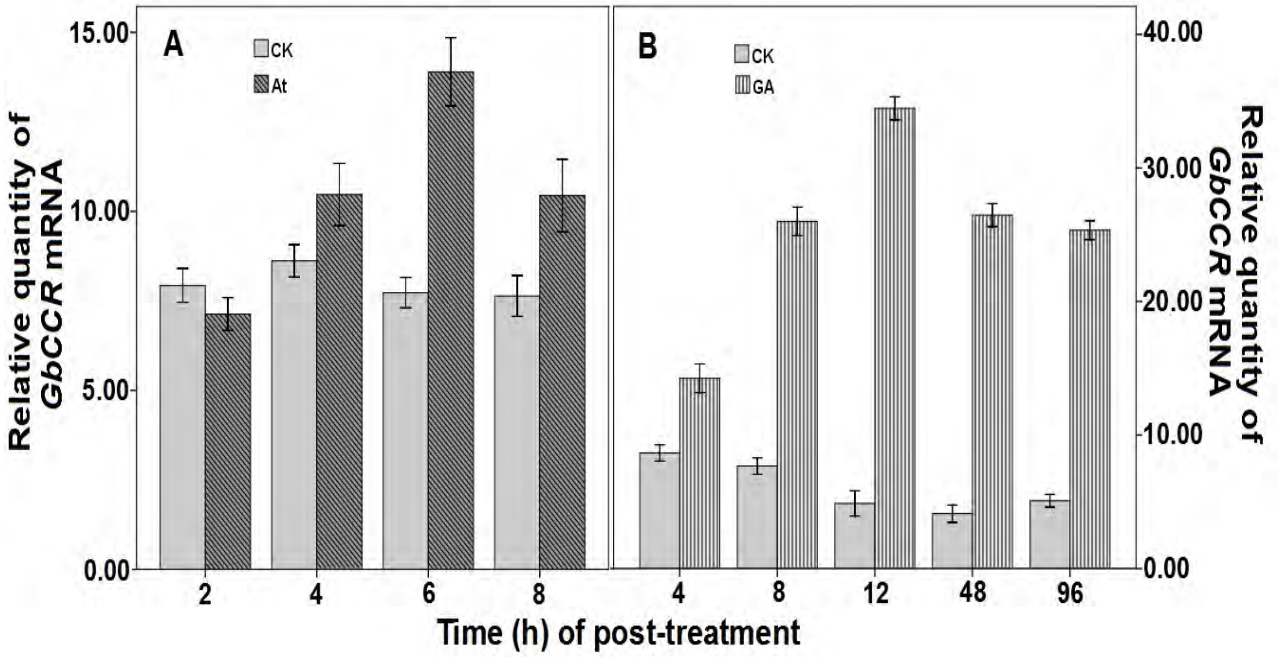

Figure 7. Relative quantities of *GbCCR* mRNA at various time points post-treatment with AT (*Agrobacterium tumefaciens*) and GA (gibberellin). Each sample was individually assayed in triplicate. Values shown represent the mean of three treated plants and the error bars indicate the standard errors of the mean.

4 h. The increasing trend continued until 6 h, when the maximum value (about 1.8 times of the control group) was reached. The expression level eventually dropped to about 1.3 times of the contrast group (Figure 7A).

When compared with *A. tumefaciens*, a higher *CCR* gene expression was obtained by treating the suspended culture cells of *Ginkgo* with GA_3. After 0.1 mM GA_3 was mixed with the suspended culture, the transcription level of *GbCCR* started to increase rapidly at 4 h to three times the expression level of the contrast group at 8 h. At 12 h, six times of the contrast group was reached, and then the value slightly decreased. However, the level of expression was still relatively high (Figure 7B).

Regulation of lignin metabolism in *Ginkgo* by *CCR*

The present study indicates that CCR is the first limiting enzyme in the catalyzing path of lignin metabolism. CCR could also regulate the carbon flow in the biosynthetic path of lignin. The relationship between the lignin content of *Ginkgo* stem and the expression level of the *GbCCR* gene was examined next. The annual cyclic change in CCR activity, *GbCCR* expression level and lignin content during the growth of *Ginkgo* stems were investigated. The results are shown in Figure 8. The first-year stems of *Ginkgo* were collected at 16 sampling points: 4th May, 18th May, 13th June, 4th July, 18th July, 27th July, 1st August, 13th August, 30th August, 11th September, 29th September, 17th October, 1st November, 14th November, 29th November and 10th December. QRT-PCR of *GbCCR*, CCR enzymatic activity and lignin content analyses were done.

Figure 8A shows the peaks of lignin content during its annual cycle. The peaks on 13th June, 11th September and 10th December appeared at 32.41, 34.81 and 33.54, respectively. The highest peak appeared on 11th September. The total lignin content has an increasing trend. CCR activity has two apparent peaks during the annual growth cycle of the first-year *Ginkgo* stems (Figure 8A). CCR activity started to increase after Ginkgo leaf buds began to unfold. The first peak appeared at about 27th July (2.37 ± 0.43 nkat·mg^{-1} protein), and is also the highest value of the year. GbCCR activity dropped on 1st August, and rapidly rose to the second peak (2.30 ± 0.29 nkat·mg^{-1} protein) on 13th August. CCR activity then dropped rapidly and reached the third small peak (0.72 ± 0.01 nkat·mg^{-1} protein), maintaining this steady trend until it slightly dropped at the last sampling point.

GbCCR expression level has three peaks during the growth of *Ginkgo* (Figure 8B). The first transcription peak appeared on 4th July, which is also the highest annual peak (21.24). The first valley appeared on 18th July, and rose to the second peak on 27th July with the relative expression of 19.43. This peak continued to drop on the 1st and 13th August, before rapidly rising to the third peak on 30th August, with the relative expression of 19.74. The activity continued to drop thereafter.

The data obtained are analyzed by linear regression. The results show that lignin content (Y_1) has a quadratic correlation with CCR activity (X_1). The concomitant

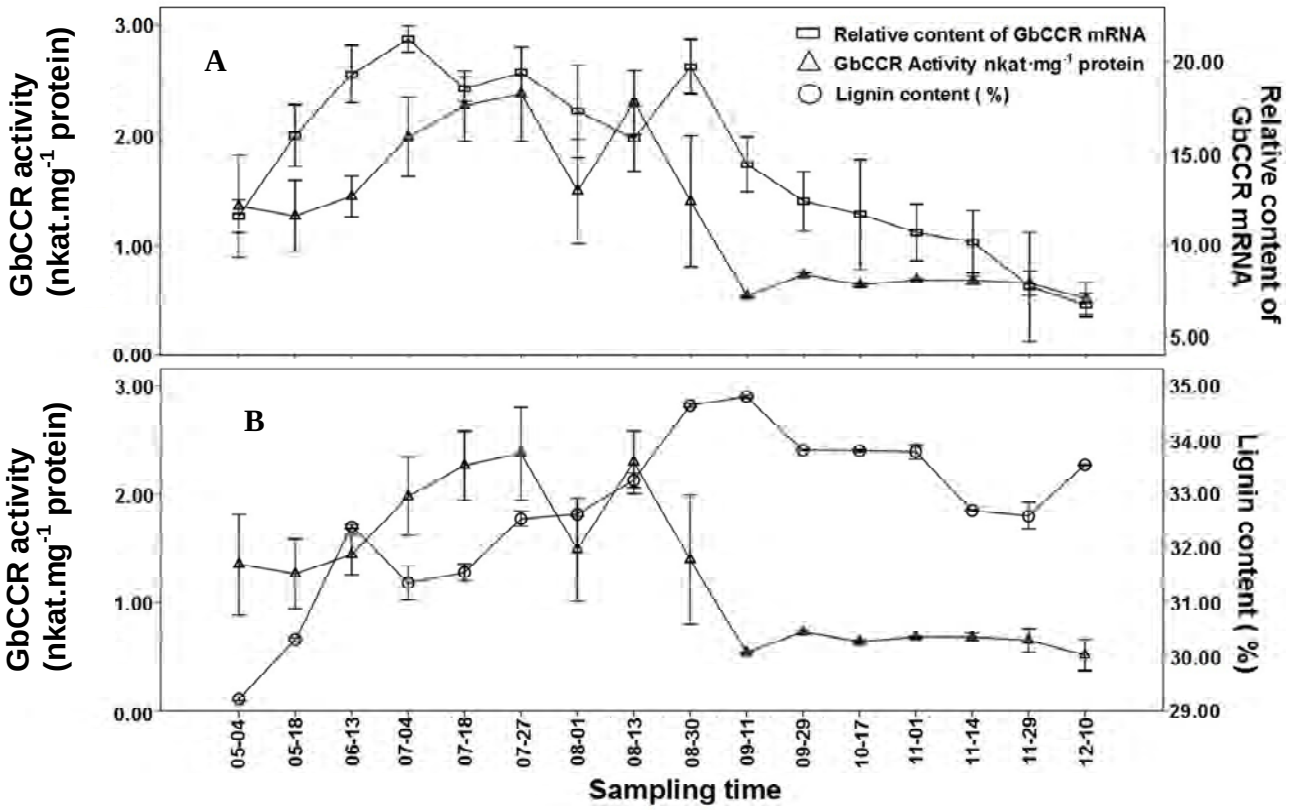

Figure 8. Changes in lignin content, GbCCR expression and activity during growth of *Ginkgo* stems, the curve for content of expression, calculated from regression equation based on CCR activity which agrees with the experimental data of lignin accumulation.

Table 3. Relationship between lignin content and CCR activity.

Model	Model summary					
	Unstandardized coefficients[a]			Quadratic regression		
	Regression coefficient	Standard Error	Significance	Multiple correlation coefficient	The coefficient of determination	Adjusted coefficient of determination
Constant	36.466	0.929	0.000			
CCR activity	-5.773	1.556	0.001	0.552	0.305	0.274
CCR activity*2	1.735	0.539	0.002			

[a]Dependent variable: Lignin contents.

probability rate is 0.002 < 0.01. The correlation coefficient is R^2 = 0.552. The correlation curve equation is as follows (Table 3): $Y_1 = (1.735 \pm 0.539) X_1^2 - (5.773 \pm 1.556) X_1 + 36.466$. Although, the change in CCR activity does not have a perfect linear relationship with the total lignin accumulation, the two are still closely correlated. The change in CCR activity (Y_2) has some linear correlation with the expression level of *GbCCR* mRNA (X_2), but was not significant. The concomitant probability p = 0.000 < 0.001. The correlation coefficient is R^2 = 0.761. The linear equation is $Y_2 = (0.113 \pm 0.014) X_2 - (0.376 \pm 0.216)$ (Table 4). The annual cyclic change in CCR activity in Ginkgo leaves is affected by the transcription level of the *CCR* gene.

DISCUSSION

Characterization and function analysis of *GbCCR*

The phenylpropane pathway can produce great secondary metabolite, including flavonoid, anthocyanin, lignin, phytoalexin and proanthocyanidins (condensed tannins). These substances play varied and important roles in

Table 4. Relationship between *CCR* expression level and activity.

Model	Model summary					
	Unstandardized Coefficients[a]			Linear regression		
	Regression coefficient	Standard Error	Significance	Multiple correlation coefficient	The coefficient of determination	Adjusted coefficient of determination
Constant	-0.376	0.216	0.088	0.761	0.580	0.571
mRNA	0.113	0.014	0.000			

[a]Dependent variable: CCR activity.

growth, disease resistance and stress tolerance in plants. The biosynthetic pathway of lignin is downstream of the general phenylpropane pathway and is parallel to other biosynthetic branches of the phenylpropane pathway, including that for flavonoid synthesis. Thus, substrate can be diverted to one pathway or the other under different conditions by changes in the activities of specific enzymes.

The *GbCCR* genes in *Ginkgo* were cloned by a simple combination of PCR and RACE technologies. Phylogenetic analysis revealed that GbCCR branched off earlier in evolution than the other cloned species-specific CCRs. During plant evolution, the flavonoid metabolic pathway acquired cinnamoyl CoA through the common phenylpropane pathway. Chalcone, flavanone and flavanol appear among ancestor species of Bryophyta, Musci plants. Chacone synthase (CHS) is the first key enzyme in the flavonoid biosynthetic pathway and appear early in plant evolution (Stafford, 1991; Koes et al., 1994). Although the first appearance of lignin (time and ancestral plant) is not known, it can be ascertained that lignin and xylan appeared in ancient pteridophyte plants (such as ferns) and can be traced back to the Devonian period (Lewis and Davin, 1994). Chacone synthase and CCR have a common substrate, ρ-courmarinyl CoA. Cinnamoyl-CoA reductase appears at the beginning of the lignin special pathway. The presence of the final products of CCR and DFR indicate that both appeared first in pteridophyte plants with vascular bundles. Cinnamoyl-CoA reductase and DFR might have originated from a common ancestor based on the comparison between the sequences of cloned CCR proteins and DFRs, and they both appeared after CHS. During evolution from bryophyte to vascular plants, the appearance of CCR served as a potential regulator of carbon flow from the phenylpropane pathway to lignin biosynthesis (Lacombe et al., 1997). The appearance of *CCR* provided the requisite conditions for the evolution and birth of xylophyta. The high content of leaf flavonoid in *G. biloba*, an ancient gymnosperm, may have arisen as concomitant with the lignin synthesis pathway controlled by *CCR* genes.

The protein sequence of many plant CCRs contain a conserved motif “KNWYCYGK” that may be within the catalytic site or the binding site for the cofactor NADPH (Larsen, 2004a, b). In some plants, this site has mutated. In rice for example, KNWYCYGK is changed to KNLYCCAK (Bai et al., 2003); while maize ZmCCR1 and ZmCCR2 evolved into RNWYCYGK and QNWYCYAK. The changes in this conserved segment might be related to substrate affinity (Pichon et al., 1998). In *Ginkgo*, there is only a single amino acid substitution to KNWYCYAK, indicating that GbCCR evolved quite early and has maintained the early sequence features of the CCR gene. At present, there are still few reports on the secondary structure of the CCR proteins in most species. In wheat, the secondary structure consists mainly of alpha helices (40.8%), random coil (29%) and only 10.7% β-folded domains (Ma and Tian, 2005). This model is quite close to GbCCR.

Cinnamoyl-CoA reductase gene belongs to a multi-gene family. It has been demonstrated through Southern blot analysis that the genomes of most plants have two or more *CCR* genes. Eight *CCR* genes are found in poplar (Li et al., 2005), there are exceptions, however. Only one *CCR* gene has been found in rice (Bai et al., 2003). Multiple homologous genes normally regulate plant metabolism through distinct temporal and spatial expression patterns and by environmentally induced expression. For example, *Arabidopsis thanliana* has two genes, *AtCCR1* and *AtCCR2*. The *AtCCR1* isoform has no regulatory role in fungal induction and is highly expressed in stems and other tissues. The *AtCCR2* isoform is generally not constitutively expressed but is induced when the plant is attacked by pathogens (Lauvergeat et al., 2001). Two homologous genes are also found in maize, *ZmCCR1* and *ZmCCR2*. The *ZmCCR1* isoform is constitutively expressed while *ZmCCT2* is induced (Pichon et al., 1998). In *Panicum virgatum*, *PvCCE1* is mainly responsible for synthesis of lignin during plant development and growth, while *PvCCR2* is upregulated in response to disease and pest attacks (Escamilla et al., 2010). The multiple CCR copies in *Ginkgo* might also have distinct functions in tissue-specific growth and defense against pathogens or environmental stress. Based on the information available, the expression of *GbCCR* is most likely induced by disease, pests and structural damage, which require further investigation.

Kinetic analysis of bacterially-expressed and purified GbCCR protein against three possible substrates showed that this enzyme preferred feruloyl CoA, and caffeoyl CoA over sinapoyl CoA. This is quite similar with Ta-CCR2,

which can convert three substrates with almost similar efficiency (Ma and Tian, 2005). In *Panicum virgatum*, recombinant PvCCR1 is active towards these substrates, but clearly prefers feruloyl CoA, whereas PvCCR2 prefers caffeoyl CoA. Based on its properties, PvCCR1 is probably associated with lignin biosynthesis during plant development, whereas PvCCR2 may function in defense (Escamilla et al., 2010). It is proposed that GbCCR enzymes may have two functions both in lignin biosynthesis and defense.

The pattern of *GbCCR* expression in different tissues

Most CCR genes show differential tissue expression. In *Arabidopsis*, *CCR1* was highly expressed in stems, while the lowest *CCR1* expression was found in leaves and flowers (Li et al., 2005). In *Eucalyptus gunnii*, *CCR* genes are also highly expressed in mature stems, followed by roots and young stems. Expression in cotyledon and euphylla are low and relatively uniform. Expression is barely detectable in the seeds of most species. Histochemistry analysis in *Eucalyptus* revealed that *CCR* genes related to lignification were mainly distributed in tissues of xylogen, phloem and cambium (Lacombe et al., 2000). In *Saccharum officinarum*, *CCR* genes showed highest expression in stems, followed by roots, while there was almost no expression in leaves (Selman et al., 1999). In *Ginkgo*, the CCR gene is most highly expressed in stems where lignin synthesis is quite active. It can be inferred that this *CCR* gene is mainly responsible for the synthesis of lignin in tissues and cell walls. In *Leucaena leucocephala*, *in situ* hybridization results indicated that the *Ll-CCR* mRNA content was highest in roots and stems, while almost no hybridization signals could be detected in leaves. It has also been shown through QRT-PCR and ELISA detection that the accumulation of lignin in stems and roots is correlated with *CCR* gene expression (Srivastava et al., 2010). The *PpCCR* transcript could be detected in leaves, flowers and fruits. In addition, *PpCCR* expression profile during fruit development were compared between two cultivars and a significant difference was found in stone cell content (Lu et al., 2010). Northern blot analysis indicated that *Ta-CCR1* was highly expressed in stem, with lower expression in leaves, and undetectable expression in roots (Ma, 2007). According to the study of Lacombe et al. (1997), the transcription of *CCR* genes in *Eucalyptus* was usually quite active in lignified tissues like leaves, stems and roots. *In situ* hybridization also demonstrated that EuCCR was expressed in the xylogen of stems but not in secondary phloem fiber or periderm. Cinnamoyl-CoA reductase expression was enhanced in soybean syncitia cells isolated by laser-microdissection from root tissue infected with the soybean cyst nematode as revealed by a *CCR* gene expression chip (Klink et al., 2009). Furthermore, *GmCCR* RNA expression was specifically induced by local or systemic wounding, drought, high salinity or by ultra-violet stress. Thus, *GmCCR* may be involved in the resistance mechanism against both biotic and abiotic stress in plants (So et al., 2010).

Through the study of the evolution of the plant vascular bundle, it has been found that the intermediary substances in the formation of plant lignin are related to plant resistance to bacteria, fungi and other pathogenic microorganism (Raven, 1977; Durner et al., 1997) and that lignification of local tissues is related to mechanical damage or attack by pathogens (Dixon, 2001). In *Arabidopsis*, *AtCCR2* does not participate in the constitutive expression of lignin, but participates in the synthesis of phenolic compounds (such as plant antitoxin) or the formation of lignin to promote plant resistance against disease, stress, or both (Lauvergeat et al., 2001). *Ginkgo* has strong resistance to diseases and pests. The expression of the *GbCCR* gene is induced by *Agrobacterium*, but the effect of induction is not significant. During the natural growth of *Ginkgo*, CCR enzyme activity and lignin synthesis are both enhanced in stems that are attacked by diseases, fungi or mechanical injury, but only physical damage induces *GbCCR* transcription. This implies that the *GbCCR* gene belongs to the constitutively expressed subgroup, and may express in response to disease.

Lignin participates in the lignification of the cell wall, increases the firmness or compression strength of cell walls, and maintains plant shape by facilitating the formation of tissues with high mechanical strength. Gibberellin promotes the rapid increase of CCR expression in suspended cell cultures of *Ginkgo*, most likely to provide cell wall materials for rapid proliferation cells. In *I. indigotica*, 1 mM GA_3 induced the expression of *CCR* genes, by expressing peaking at 2 h (Hu et al., 2011). Interestingly, the flavone content of *Ginkgo* was enhanced by lower GA concentrations (Cheng et al., 2004). We inferred that the slow growth of *Ginkgo* timber might result from the redirection of substrates away from lignin biosynthesis and toward flavonoid biosynthesis in *Ginkgo* leaves.

The spatio-temporal expression patterns of the *GbCCR* gene and accumulation of lignin in annual stem developmental stages

Accumulating evidences indicates that *CCR* expression is correlated with lignin accumulation in plants (Kawasaki et al., 2006; Van Der et al., 2006). In *Leucaena leucocephala*, QRT-PCR and ELISA assays revealed that the accumulation of lignin in stems and roots was correlated with *CCR* gene and protein expression (Srivastava et al., 2010). In two different strains of wheat (H4564 and C6001), *Ta-CCR1* expression was maintained at high levels during the extension, earing and grain filling periods of H4564 stems. In the C6001 strain,

however, the transcription level dropped abruptly at earing and grain filling stages. In these two strains, total CCR enzymatic activity correlated with the transcription level of Ta-CCR1. In the strain H4564, the higher TaCCR1 transcription level and CCR total enzymatic activity were related with the content of Klason lignin and with the mechanic strength of the stem, indicating that Ta-CCR1 and CCR enzyme activity play an important role in regulating the synthesis of lignin and in improving the mechanical strength of wheat stem during maturation (Ma, 2007). Tu (2010) identified candidate genes encoding *LpCCR* and *LpCOMT* in *Lolium perenne* and demonstrated that the spatio-temporal expression patterns of these genes were correlated with the developmental profile of lignin deposition. In one-year-old *Ginkgo* stem, the transcription level of the *GbCCR* gene is directly and positively correlated with GbCCR activity and activity is in turn correlated with accumulation of lignin. Hence, the annual changes in lignin synthesis should be directly related with the transcription regulation of *GbCCR* gene, which is also consistent with GA_3-mediated induction of *GbCCR* expression. However, previous study showed that exogenous GA_3 restrained the gene expression level of flavonoids and reduction of accumulated content of *Ginkgo* flavones (Cheng et al., 2011). Therefore, GA_3 treatment is not suitable for the cultivation of *Ginkgo* flavonoids. Maybe, it is a useful target for the engineering of improved lumber.

It has been suggested that the CCR-mediated reaction products, monolignols are not only designed for polymerization into lignins, but also constitute a substrate for antioxidants or biocides (Lacombe et al., 1997). In soybean, it is not known whether *GmCCR* gene expression correlates with lignin synthesis or resistance to oxidation and disease, but one study suggested that *GmCCR* is involved in both (So et al., 2010). In light of these properties and expression pattern, we proposed that *GbCCR* is probably associated with lignin biosynthesis during plant development, at the same time, *GbCCR* also have function in defense. The expression profiling analyses of *GbCCR* under stress conditions and different tissues suggest that *GbCCR* may be involved in environmental stress and timber quality, which serves as an initial step to further study on *GbCCR* biosynthesis and improvement on the production of phenylpropanoid compounds in *G. biloba* by metabolic engineering in future. This study will enable us to further understand the role of GbCCR in the synthesis of phenylpropanoid compounds in *G. biloba* at the molecular level.

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Abbreviations

IPTG, Isopropyl β-D-thiogalactoside, $C_9H_{18}O_5S$; **PCR,** polymerase chain reaction; **RACE,** rapid amplification of cDNA ends; **QRT-PCR,** quantitative real-time polymerase chain reaction; **RT-PCR,** reverse transcriptase polymerase chain reaction; **S.D.,** standard deviation ; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **ORF,** open reading frame; **MS,** Murashige and Skoog culture medium.

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